Physiol. Res. 47: 501–505, 1998

The Influence of Angiotensin II on Sex-Dependent Proliferation of Aortic VSMC Isolated from SHR

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Received October 1, 1998
Accepted November 30, 1998

Summary

The growth response to angiotensin II (Ang II) was studied using cultured vascular smooth muscle cells (VSMC) isolated from the aortae of male and female spontaneously hypertensive rats (SHR). Systolic and mean arterial blood pressure of 10-week-old males was significantly higher when compared to age-matched females. The specific growth rate of male VSMC was significantly higher on the third and sixth day after synchronisation. Angiotensin II in concentration 10^{-7} M stimulated the specific growth rate only in male VSMC during the exponential phase of growth. Moreover, doubling time was 3 hours shorter in male VSMC in comparison with the females. Our results suggest that both the increased specific growth rate and augmented growth-response of male VSMC to Ang II may explain the higher sensitivity of males to hypertensive stimuli.

Key words

Spontaneously hypertensive rat – Gender – Tissue culture – Aortic smooth muscle cells – Angiotensin II

Introduction

Sex-dependent differences in the prevalence of hypertension have been reported in humans. The data from the Framingham study indicated that the morbidity and mortality due to cardiovascular diseases in women with normal blood pressure was lower by 50 % than in normotensive men (Vokonas *et al.* 1988).

It is evident that growth of vascular smooth muscle cells (VSMC) plays an important role in the pathogenesis of hypertension (Schwartz *et al.* 1986). Although much is known about growth abnormalities of VSMC in hypertension (Owens and Schwartz 1982, Liu *et al.* 1988), the factors that initiate and perpetuate the hypertension-related increase in the mass of vascular media are not completely understood.

As has been shown by several authors, the proliferation rate of VSMC is higher in SHR compared to normotensive Wistar-Kyoto (WKY) rats (Yamori *et al.* 1981, Hadrava *et al.* 1989). Furthermore, cultured VSMC from SHR aortae have been observed to proliferate more rapidly in response to various growth factors including angiotensin II (Ang II) than cells isolated from normotensive animals (Hadrava *et al.* 1989, Paquet *et al.* 1990). Experimental data from normotensive rats (Travo *et al.* 1980, Bačáková *et al.* 1997) have demonstrated that aortic VSMC isolated from male Wistar or Wistar-Kyoto rats proliferate more

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rapidly than those isolated from females. Moreover, we have observed that this sex-dependent difference in VSMC growth also exists when cells are isolated from the aortae of SHR (Bačáková and Kuneš 1995).

Angiotensin II, the final effector peptide of the renin-angiotensin system, is both a pressor hormone and a trophic peptide (Schelling *et al.* 1991, Owens 1989). Its growth promoting activity for various cell types including VSMC was well documented (Owens and Schwartz 1982, Wolf *et al.* 1996). To our knowledge, no data about the different reactivity of male and female VSMC to angiotensin II are available.

This study was designed to further analyse the difference in VSMC growth isolated from male and female SHR. Therefore, the responsiveness of VSMC to Ang II was evaluated in both genders.

Material and Methods

Male (n=8) and female (n=8) SHR aged 10 weeks were obtained from the breeding colony of our institute. The rats were killed by decapitation and VSMC were obtained by enzymatic digestion from their thoracic aortae. Briefly, thoracic aortae were aseptically excised and cleaned of connective tissue and adherent fat. The adventitia was removed by stripping the aortae with a pair of dissecting forceps. Isolated arteries were cut longitudinally and the endothelium was removed. The medial segments were rinsed with Dulbecco's Modified Eagle Medium (DMEM) plus gentamycin, minced into very small pieces and digested using collagenase 2 mg/ml (Sigma), elastase 0.12 mg/ml (Sigma) and bovine serum albumin 2 mg/ml (Sigma) at 37 °C for 60 min (Touyz *et al.* 1994). Isolated cells were centrifuged and resuspended in DMEM (SEVAC, Prague) + 20 % foetal calf serum + gentamycin. Cells were plated in 25 cm^2 flasks (Nunclon) and maintained at 37 °C in a humidified incubator in 95 % air to 5 % CO_2 atmosphere till the confluence. Confluent cells exhibited a hill-and-valley pattern typical for VSMC in culture. Passaged cells were positively identified as VSMC by immunofluorescence using α-actin antibody. Cells from passages 2–10 were used in this study.

After trypsinization using 0.05 % trypsin and 0.04 % EGTA, cells were counted by Coulter Counter and seeded into 24-well dishes at the density of $4x10^4$ cells/well. After 24 h, the cells were rendered quiescent by serum deprivation for 42 h. Quiescent cells were exposed to angiotensin II (Hypertensin, Ciba, 10^{-7} M) for 1, 3 or 6 days. At the end of incubation, cells were harvested by trypsinization and counted by Coulter Counter. The specific growth rate was determined according to the formula $(N_2 - N_1)/(N_1 (t_2 - t_1))$, where N_1 and N_2 are cell numbers at times t_1 and t_2 (in days). The doubling time was calculated according to the formula $\log 2 (t_2 - t_1)/(\log N_2 - \log N_1)$.

Student's t-test was used for comparison of both genders. The paired t-test was employed for evaluation of the Ang II effect. Results from five independent experiments in triplicates were expressed as means ± S.E.M. A value of $p<0.05$ was considered to be significant.

Results

Table 1 summarises body weight and blood pressure values of male and female spontaneously hypertensive rats whose aortae were used for isolation of VSMC. Both systolic and mean arterial blood pressures were significantly higher in males than in females.

Table 1
Body weight (BW), systolic (SBP), mean arterial (MAP and diastolic (DBP) blood pressure in 10-week-old male and female spontaneously hypertensive rats.

	Males n=8	Females n=8
BW (g)	235±7	167±3**
SBP (mm Hg)	194±4	176±3**
MAP (mm Hg)	159±3	145±5*
DBP (mm Hg)	129±3	118±6

*Data are means ± S.E.M., *p<0.05, **p<0.005, significantly different from males.*

To examine if there is a growth difference between the cells from the two genders, cells were

harvested 1, 3 or 6 days after synchronisation. The specific growth rate was the same in control cells (without stimulation by Ang II) of both genders on day 1 (Table 2). A significantly higher specific growth rate was attained by VSMC from males as compared with those from females on day 3 and persisted until day 6. There was no effect of Ang II on the specific growth rate in both genders except for the male VSMC in which Ang II stimulated the growth rate on day 3 significantly.

Moreover, the doubling time determined from the growth curves of exponentially proliferating 1- to 3-day-old cultures was about three hours shorter in male VSMC in comparison with female cells (26.1 ± 0.7 h vs 29.0 ± 1.3 h, p=0.06).

Table 2
Effect of time and angiotensin II (10^{-7} M) on specific growth rate of VSMC isolated from male and female SHR.

Time (t_1–t_2)	Ang II	Male	Female
0 – 1	–	0.95±0.12	0.84±0.10
	+	0.97±0.14	0.88±0.10
0 – 3	–	1.99±0.10	1.63±0.12*
	+	2.17±0.15$^{\#}$	1.72±0.14*
0 – 6	–	1.48±0.07	1.00±0.09**
	+	1.43±0.03	0.95±0.08**

*Data are means ± S.E.M. VSMC without stimulation by Ang II (–) were compared to cells stimulated by 10^{-7} M Ang II (+). *p<0.05, **p<0.01 as compared with males, $^{\#}$p<0.05 as compared with controls.*

Discussion

The present study in vascular smooth muscle cells isolated from spontaneously hypertensive rats shows a significantly higher specific growth rate of male VSMC when compared to female cells. This is in good agreement with our previous results (Bačáková and Kuneš 1995). The same sex difference in VSMC growth was observed for cells isolated from normotensive rats (Travo *et al.* 1980, Bačáková *et al.* 1997). The mechanisms responsible for sex-dependent differences in VSMC proliferation are still not well clarified. Among the factors that control these differences *in vivo*, sex hormones are believed to be of a considerable importance. Receptors for 17β oestrogen are present on both vascular endothelium and smooth muscle cells (Horwitz and Horwitz 1982, Hanes *et al.* 1996). It was shown that oestrogen inhibits intimal thickening of the rat femoral artery in females and this was reversed by ovariectomy (Akishita *et al.* 1997). Gender differences in vascular contractility have also been reported by several investigators (Stallone *et al.* 1991, Zhang *et al.* 1992). Moreover, there is *in vitro* evidence that oestrogens slow down VSMC proliferation (Chang *et al.* 1980), whereas testosterone may support it (Masuda *et al.* 1991). However, the role of sex hormones in our study seems to be negligible, because the foetal calf serum added to the culture medium contained testosterone or oestradiol in concentrations less than 10^{-10} M (Bačáková *et al.* 1997). This concentration is several orders of magnitude lower than that required for inducing growth effects *in vitro* (Chang *et al.* 1980, Masuda *et al.* 1991). The reason for the different specific growth rate of VSMC isolated either from male or female SHR needs to be further analysed.

We therefore studied the specific growth rate of VSMC from both genders after the stimulation by Ang II. The specific growth rate was not influenced by Ang II except for male VSMC during their exponential phase of growth. It has been shown that Ang II, a potent vasoconstrictor, stimulates VSMC growth in the vascular wall when administered *in vivo* (Wiener *et al.* 1996, Su *et al.* 1998). On the other hand, discrepant results have been reported when Ang II is given *in vitro*, i.e. it increases either the size (Berk *et al.* 1989, Geisterfer *et al.* 1988) or the number of VSMC (Paquet *et al.* 1990). The different stimulation of male and female VSMC by Ang II in our study might be analogous. While the male

cells increased their specific growth rate during the exponential phase of growth, suggesting that hyperplasia is enhanced, the female cells did not respond to Ang II significantly. However, this does not mean that female cells could not react by specific hypertrophy without cell division because we did not measure protein synthesis in our experiments. Ang II at the concentration 10^{-6} M induced a weak hyperplastic response in VSMC from Wistar rats, but a clear-cut increase in the number of VSMC from SHR (Virone-Oddos *et al.* 1997). Furthermore, the concentration $5x10^{-7}$ M caused maximal stimulation of protein synthesis in cells of Wistar rats, while the same effect in SHR cells was reached at a concentration $5x10^{-8}$ M. Recently, bimodal effects of Ang II on migration of both human and rat VSMC were also observed (Liu *et al.* 1997). In the light of these results, it is evident that a detailed analysis of the different reactivity of male and female VSMC to Ang II should be carried out.

In conclusion, the different reactivity of male and female VSMC to angiotensin II found in our study might contribute to the higher sensitivity of males to hypertensive stimuli.

Acknowledgements

This work was supported by grant 306/96/1283 from Grant Agency of the Czech Republic.

References

AKISHITA M., OUCHI Y., MIYOSHI H., KOZAKI K., INOUE S., ISHIKAWA M., ETO M., TOBA K., ORIMO H.: Estrogen inhibits cuff-induced intimal thickening of rat femoral artery: effects on migration and proliferation of vascular smooth muscle cells. *Atherosclerosis* **130:** 1–10, 1997.

BAČÁKOVÁ L., KUNEŠ J.: Sex-dependent differences in growth of vascular smooth muscle cells from spontaneously hypertensive rats. *Physiol. Res.* **44:** 127–130, 1995.

BAČÁKOVÁ L., MAREŠ V., LISÁ V., BOTTONE M.G., PELLICCIARI C., KOCOUREK F.: Sex related differences in the adhesion, migration and growth of rat aortic smooth muscle cells in culture. *In Vitro Cell. Dev. Biol.* **33:** 410–413, 1997.

BERK B.C., VEKSHTEIN V., GORDON H.M., TSUDA T.: Angiotensin II-stimulated protein synthesis in cultured vascular smooth muscle cells. *Hypertension* **13:** 305–314, 1989.

CHANG C.W., NAKAO J., ORIMO H., MUROTA S.I.: Stimulation of prostacyclin biosynthetic activity by estradiol in rat aortic smooth muscle cells in culture. *Biochim. Biophys. Acta* **619:** 107–118, 1980.

GEISTERFER A.A., PEACH M.J., OWENS G.K.: Angiotensin II induces hypertrophy, not hyperplasia, of cultured rat aortic smooth muscle cells. *Circ. Res.* **62:** 749–756, 1988.

HADRAVA V., TREMBLAY J., HAMET P.: Abnormalities in growth characteristics of aortic smooth muscle cells in spontaneously hypertensive rats. *Hypertension* **13:** 589–597, 1989.

HANES D.S., WEIR M.R., SOWERS J.R.: Gender considerations in hypertension pathophysiology and treatment. *Am. J. Med.* **101:** 10S–21S, 1996.

HORWITZ K.B., HORWITZ L.D.: Canine vascular tissues are targets for androgens, estrogens, progestins, and glucocorticoids. *J. Clin. Invest.* **69:** 750–758, 1982.

LIU G., ESPINOSA E., OEMAR B.S., LÜSCHER T.F.: Bimodal effects of angiotensin II on migration of human and rat smooth muscle cells. Direct stimulation and indirect inhibition via transforming growth factor-β1. *Arterioscler. Thromb. Vasc. Biol.* **17:** 1251–1257, 1997.

LIU J.L., BISHOP S.P., OVERBECK H.W.: Morphometric evidence for non-pressure-related arterial wall thickening in hypertension. *Circ. Res.* **62:** 1001–1010, 1988.

MASUDA A., MATHUR R., HALUSHKA P.V.: Testosterone increases thromboxane A_2 receptors in cultured rat aortic smooth muscle cells. *Circ. Res.* **69:** 638–643, 1991.

OWENS G.K.: Control of hypertrophic versus hyperplastic growth of vascular smooth muscle cells. *Am. J. Physiol.* **257:** H1755–H1765, 1989.

OWENS G.K., SCHWARTZ S.M.: Alterations in vascular smooth muscle mass in the spontaneously hypertensive rat. Role of cellular hypertrophy, hyperploidy, and hyperplasia. *Circ. Res.* **51:** 280–289, 1982.

PAQUET J.L., BAUDOUIN-LEGROS M., BRUNELLE G., MEYER P.: Angiotensin II-induced proliferation of aortic myocytes in spontaneously hypertensive rats. *J. Hypertens.* **8:** 565–572, 1990.

SCHELLING P., FISCHER H., GANTEN D.: Angiotensin and cell growth: a link to cardiovascular hypertrophy? *J. Hypertens.* **9:** 3–15, 1991.

SCHWARTZ S.M., CAMPBELL G.R., CAMPBELL J.H.: Replication of smooth muscle cells in vascular disease. *Circ. Res.* **58:** 427–444, 1986.

STALLONE J.N., CROFTON J.T., SHARE L.: Sexual dimorphism in vasopressin-induced contraction of rat aorta. *Am. J. Physiol.* **260:** H453–H458, 1991.

SU E.J., LOMBARDI D.M., SIEGAL J., SCHWARTZ S.M.: Angiotensin II induces vascular smooth muscle cell replication independent of blood pressure. *Hypertension* **31:** 1331–1337, 1998.

TOUYZ R.M., TOLLOCZKO B., SCHIFFRIN E.L.: Mesenteric vascular smooth muscle cells from spontaneously hypertensive rats display increased calcium responses to angiotensin II but not to endothelin-1. *J. Hypertens.* **12:** 663–673, 1994.

TRAVO P., BARRETT G., BURNSTOCK G.: Differences in proliferation of primary cultures of vascular smooth muscle cells taken from male and female rats. *Blood Vessels* **17:** 110–116, 1980.

VIRONE-ODDOS A., DESANGLE V., PROVOST D., CAZES M., CAUSSADE F., CLOAREC A.: In vitro and in vivo effects of UP 269-6, a new potent orally active nonpeptide angiotensin II receptor antagonist, on vascular smooth muscle cell proliferation. *Br. J. Pharmacol.* **120:** 488–494, 1997.

VOKONAS P.S., KANNEL W.B., CUPPLES L.A.: Epidemiology and risk of hypertension in the elderly: the Framingham study. *J. Hypertens.* **6** (Suppl. 1): S3–S9, 1988.

WIENER J., LOMBARDI D.M., SU J.E., SCHWARTZ S.M.: Immunohistochemical and molecular characterization of the differential response of the rat mesenteric microvasculature to angiotensin-II infusion. *J. Vasc. Res.* **33:** 195–208, 1996.

WOLF G., ZIYADEH F.N., ZAHNER G., STAHL R.A.: Angiotensin II is mitogenic for cultured rat glomerular endothelial cells. *Hypertension* **27:** 897–905, 1996.

YAMORI Y., IGAWA T., KANBE T., KIHARA M., NARA Y., HORIE R.: Mechanisms of structural vascular changes in genetic hypertension: analyses on cultured vascular smooth muscle cells from spontaneously hypertensive rats. *Clin. Sci.* **61** (Suppl. 7): 121s–123s, 1981.

ZHANG A.M., ALTURA B.T., ALTURA B.M.: Endothelial-dependent sexual dimorphism in vascular smooth muscle: role of Mg^{2+} and Na^{+}. *Br. J. Pharmacol.* **105:** 305–310, 1992.

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